Acetylcholine Receptor in a C2 Muscle Cell Variant Is Retained in the Endoplasmic Reticulum

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Abstract. We have examined the properties and intracellular localization of acetylcholine receptors in the C2 muscle cell line and in a variant (T^-) that accumulates AChR intracellularly. On immunoblots, the subunit structures of the AChR from wild-type and T^- cells were similar except that the γ and δ subunits of the variant AChR had altered mobilities. Digestion with endoglycosidases H and F demonstrated that this difference results from a failure of high-mannose N-linked oligosaccharides on AChR subunits to be processed to complex forms in the variant. N-linked glycosylation of other proteins in the variant was normal. When examined by immunocytochemistry, the distribution of internal AChR in wild-type cells was consistent with a location both in the endoplasmic reticulum and in the Golgi. Variant cells, however, showed no evidence of Golgi staining. Subcellular fractionation experiments also demonstrated AChR in the Golgi fractions of wild-type cells, but not in those derived from T^- cells. We conclude that in T^- myotubes most of the AChR fails to be transported out of the endoplasmic reticulum.

MEMBRANE proteins follow a common path of assembly and transport to the cell surface (reviewed in Burgess and Kelly, 1987; Pfeffer and Rothman, 1987). They are synthesized, glycosylated and assembled in the ER, transported to the Golgi apparatus where N-linked oligosaccharides are processed and other modifications made, and then transported to the cell surface. Proteins may deviate from this pathway under a variety of circumstances. Misfolding of the protein or failure to assemble can result in retention in the ER (Doyle et al., 1985, 1986; Copeland et al., 1986, 1988; Gething et al., 1986; Kreis and Lodish, 1986; Salter and Cresswell, 1986; Doms et al., 1988; Chen et al., 1988). Experimental manipulations such as change in pH, reduced temperature, or the addition of ionophores can also disrupt the movement of proteins to the surface, resulting in their intracellular retention (Matlin et al., 1988; Strous et al., 1985; Matlin, 1986; Griffiths et al., 1983; Matlin and Simons, 1983; Saraste and Kuismanen, 1984). These deviations from the normal pathway are useful for its dissection and for attempts to understand its regulation.

The nicotinic acetylcholine receptor (AChR)[1] is an oligomeric membrane protein expressed in differentiated muscle cells that follows a pathway to the cell surface resembling that of other membrane proteins (Anderson and Blobel, 1981, 1983; Merlie et al., 1983; Carlin and Merlie, 1987). The receptor is assembled in the ER from four highly homologous glycosylated subunits to form the structure $\alpha_2\beta\gamma\delta$ (Smith et al., 1987). It is then transported to the Golgi apparatus (Fambrough and Devreotes, 1978), where some of the oligosaccharides on the γ and δ subunits are processed to complex forms (Gu and Hall, 1988*b*), and the fully processed AChR is then transported to the cell surface (Devreotes et al., 1977; Merlie et al., 1983).

We have recently investigated a genetic variant of the C2 mouse muscle cell line, T^-, that expresses a reduced amount of AChR on its surface (Black and Hall, 1985). Previous experiments (Gu et al., 1989) have shown that, although the T^- variant synthesizes a normal amount of receptor, only a small fraction of the assembled AChR reaches the cell surface. The remainder is accumulated in the cell interior. Here we investigate the molecular properties and intracellular location of the AChR in wild-type and variant C2 myotubes to determine the point in the intracellular pathway to the surface at which the AChR in variant cells has been retained or diverted.

Materials and Methods

Antibodies

mAbs 61 and 124 (Tzartos et al., 1981; Gullick and Lindstrom, 1983), which recognize the α and β subunit, respectively, were generously given to us by Dr. Jon Lindstrom (The Salk Institute for Biological Studies, San Diego, CA); mAb 14-3-F7, specific for the α subunit, was prepared as described in Dowding and Hall (1987); anti-γ 485 and anti-δ 486 antibodies, which

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1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; BiP, immunoglobulin heavy chain-binding protein; endo F, endoglycosidase F; endo H, endoglycosidase H; rho-α-BuTx, rhodamine-conjugated α-bungarotoxin.

recognize the γ and δ subunit, respectively (Gu and Hall, 1988*a*,*b*), were prepared as described in Gu and Hall (1988*a*); anti-BiP (immunoglobulin heavy chain-binding protein) monoclonal antibody (Bole et al., 1986) was the generous gift of Dr. David Bole (Yale University, New Haven, CT); anti-Golgi monoclonal antibody (Allan and Kreis, 1986) was kindly provided by Dr. V. Allan (University of California, San Francisco, CA); and a polyclonal antiserum raised against the Japanese strain of influenza virus was the generous gift of Dr. Judith White (University of California, San Francisco, CA).

Cell Culture and Metabolic Labeling Conditions

The C2 mouse muscle cell line, isolated originally by Yaffe and Saxel (1977), was maintained in DME H-16 supplemented with 20% FBS and 0.5% chick embryo extract as described in Inestrosa et al. (1983) and Gu et al. (1985). Differentiation of myoblasts into myotubes was stimulated by transferring cells into fusion medium (DME H-16 supplemented with 10% horse serum). Cells were usually used 3–4 d after transfer to fusion medium.

Metabolic labeling of C2 myotubes in culture was routinely carried out with [^{35}S]methionine/cysteine (Tran ^{35}S label; ICN Radiochemicals, Irvine, CA). To label AChRs, myotube cultures in 10-cm dishes were incubated with 1 mCi [^{35}S]methionine/cysteine overnight in methionine/cysteine-free DME H-16 supplemented with 2% horse serum. To label fibronectin, cells were incubated with labeled amino acids for 6 h under the same conditions. To pulse-label hemagglutinin produced by cells infected with influenza virus, infected cells in 60-mm dishes were washed with 2 ml methionine/cysteine-free medium and incubated for 15 min. They were labeled with the same medium containing 0.5 mCi [^{35}S]methionine/cysteine for 10 min at 37°C. Incorporation of radioactive amino acids was stopped by washing the cells with 5 ml PBS containing 2 mg/ml unlabeled methionine and cysteine; the cells were then chased in 5 ml fusion medium for the indicated time.

AChR Preparation and Western Blot Analysis

AChRs were isolated from C2 myotube cultures according to the procedure previously described (Gu and Hall, 1988*b*). Myotubes were collected by scraping into PBS with a rubber policeman. The cell pellet obtained by centrifugation was then extracted with a buffer containing 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.4 mM PMSF, 1 mM *N*-ethylmaleimide (NEM), 1 mM sodium tetrathionate, 20 μg/ml leupeptin, 20 μg/ml pepstatin, 20 U/ml aprotinin (buffer A). For isolation of AChRs from metabolically labeled cultures, the extract was applied to an α-cobratoxin-Sepharose column and the bound receptor eluted with 1 M carbamylcholine. Receptor was further purified by immunoprecipitation with mAb 61, a monoclonal antibody that recognizes the α subunit of the receptor (Tzartos et al., 1981). For endoglycosidase digestion and Western blot analysis, AChRs in extracts of unlabeled cells were adsorbed to α-bungarotoxin (α-BuTx) coupled to Sepharose and eluted by boiling in 0.6% SDS; they were then treated with endoglycosidase H (endo H) or endoglycosidase F (endo F). AChRs were routinely analyzed on 9% SDS polyacrylamide gels and, for Western blot analysis, transferred to nitrocellulose membranes and probed with subunit-specific antibodies as described in Gu and Hall (1988*b*). In some experiments, receptors on the cell surface were separated from internal ones by incubating intact myotubes with unlabeled α-BuTx, followed by extraction and immunoprecipitation with anti-α-BuTx antibodies.

Isolation of Fibronectin

Fibronectin was isolated from myotube culture medium according to the method of Ruoslahti et al. (1982), using affinity chromatography on gelatin-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) as the single purification step.

Expression and Isolation of Influenza Hemagglutinin

C2 myotubes were infected with the Japanese strain of influenza virus (generously given to us by Dr. Judith White, University of California, San Francisco, CA) according to the procedure described by Yewdell et al. (1988). Confluent myotube cultures in 60-mm dishes were rinsed with 5 ml PBS and incubated at room temperature with 2 ml DME H-16 supplemented with 0.1% BSA and 20 mM Hepes (pH 7.6) containing 400 hemagglutinin (HA) units of infectious influenza virus. 5 ml of DME supplemented with 10% horse serum were then added and the incubation continued for 6 h at 37°C. Cells were then washed with PBS and labeled with [^{35}S]methionine/cysteine as described above. Pulse-chase labeled cells were collected by scraping them into PBS and the cell pellet obtained by centrifugation extracted in 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 20 U/ml aprotinin, 20 μg/ml soybean trypsin inhibitor. HA was isolated from the extract by immunoprecipitation with an antiserum raised against the influenza virus. Immunoprecipitates were washed with 1 M NaCl, 0.1% SDS, 0.1% Triton X-100, 50 mM Tris, pH 7.4 and eluted by boiling in 0.6% SDS.

Endo H and Endo F Digestion

Endoglycosidase digestion of isolated proteins was carried out as previously described (Gu and Hall, 1988*b*). For digestion of the AChR, the digestion mixture contained 0.2% SDS, 20 mM sodium citrate (pH 5.5), 10 mM EDTA, and 10 mU/ml endo H (Boehringer Mannheim Biochemicals, Indianapolis, IN) or 4 U/ml endo F (New England Nuclear, Boston, MA); for digestion of fibronectin and HA, the reaction mixture also contained 0.1 M β-mercaptoethanol. The reaction was carried out at 37°C for 15–18 h and was stopped by boiling in SDS-PAGE sample buffer.

Subcellular Fractionation of C2 Myotubes

Subcellular fractionation of C2 myotubes were carried out as described by Smith et al. (1987) for BC3H-1 cells. Wild-type or T$^-$ myotube cultures in 10-cm dishes were rinsed twice with TBS (25 mM Tris-HCl, pH 7.4, 144 mM NaCl) and collected in 0.7 ml homogenization buffer containing 2.7% sucrose, 1 mM $MgCl_2$, 5 mM K-Hepes, pH 7.5. The cells were then homogenized with seven strokes in a 7-ml Dounce homogenizer (Wheaton Instruments Div., Millville, NJ). The homogenate was loaded on a 20–69% sucrose gradient and centrifuged in a rotor (Model SW 60; Beckman Instruments Inc., Palo Alto, CA) for 30 min at 60,000 rpm as described in Smith et al. (1987). 200 μl fractions were then collected from the top of the gradient with a micropipet and each fraction assayed for AChR and for ER and Golgi markers.

Assays

The amount of AChR in sucrose gradient fractions was determined with the DEAE filtration assay described in Brockes and Hall (1975). Aliquots of gradient samples were mixed with equal volumes of 2× concentrated buffer A and incubated with ^{125}I-labeled α-BuTx (Amersham Corp., Arlington Heights, IL) for 2 h at 37°C. The reaction mixture was then applied to DEAE filters and bound receptor determined by counting in a gamma counter. Glucose-6-phosphatase, UDP-galactose/*N*-acetylglucosamine galactosyltransferase, and mannosidase II activities were measured according to the methods described in Smith et al. (1987).

Immunocytochemistry

For immunofluorescence, C2 cells were grown on round glass coverslips (gold seal, 1/4" diam) that were kept in 24-well plates. All solutions were prepared with PBS unless otherwise stated.

For staining of the intracellular AChR, the cells were incubated with 1 μM unlabeled α-BuTx for 90 min to block surface AChR. They were then rinsed with PBS, fixed with 2% paraformaldehyde for 20 min, permeabilized with 1% Triton X-100 for 10 min, and stained for 2 h with rhodamine-conjugated α-bungarotoxin (rho-α-BuTx) in PBS containing 1% BSA (PBS/BSA). They were rinsed again and mounted in glycerol supplemented with para-phenylenediamine (Platt and Michael, 1983).

For staining of the Golgi apparatus, cells were fixed as described above and permeabilized with 0.1% Triton X-100/0.5% SDS in PBS. They were incubated for 15 min in 10% FCS/4% BSA to block nonspecific binding and then for 2 h with an anti-Golgi monoclonal antibody diluted in the same buffer. This antibody recognizes a 110-kD Golgi protein and shows a pattern of staining that is very similar to that obtained with classical Golgi markers (Allan and Kreis, 1986). Finally, the cells were incubated with a fluorescein-conjugated goat anti-mouse antibody (Cappel Laboratories, Malvern, PA) in PBS/BSA, rinsed and mounted as described above.

For staining of the ER, cells were handled as described in the previous section, except that permeabilization was with 1% Triton X-100 and the blocking step was omitted. An anti-immunoglobulin heavy chain-binding protein (BiP) rat monoclonal antibody (Bole et al., 1986) was applied for 2 h in PBS/BSA, followed by fluorescein-conjugated goat anti-rat antibodies.

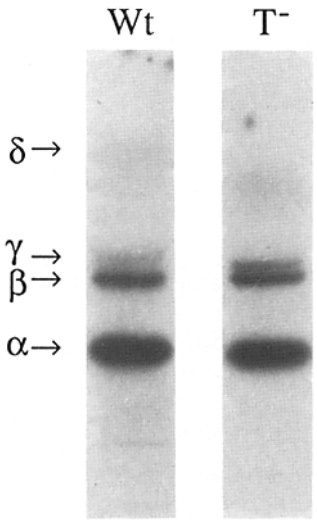

Figure 1. Subunit composition of AChRs in wild-type and T⁻ variant cells. Wild-type (*Wt*) or T⁻ myotube cultures were metabolically labeled overnight with [^{35}S]methionine/cysteine. The cells were then collected and extracted and AChRs isolated from the extract by affinity chromatography on an α-cobratoxin-Sepharose column, followed by immunoprecipitation with mAb 61 as described in Materials and Methods. Immunoprecipitates were analyzed on 9% SDS polyacrylamide gels under reducing conditions and fluorographed. The figure shows an autoradiograph of the gel. Note that the mobilities of the bands tentatively identified as the γ and δ subunits of the receptor are different in wild-type and T⁻ cells.

Results

T⁻ AChR Has Abnormal N-linked Oligosaccharides

To compare the subunit structures of the AChR in wild-type and T⁻ myotubes, we labeled myotube cultures overnight with [^{35}S]methionine/cysteine and purified the AChR by affinity chromatography on cobratoxin-Sepharose column, followed by immunoprecipitation with mAb 61, a monoclonal antibody that recognizes the α subunit (Tzartos et al., 1981). The purified AChRs were then analyzed by SDS-PAGE and fluorography. Four bands were detected in each preparation (Fig. 1). All appeared to be subunits of the AChR as they were not present in a control preparation in which the myotube extracts were incubated with α-BuTx before the cobratoxin-Sepharose purification step (data not shown). The two bands of lower apparent molecular weights corresponded to the previously identified α and β subunits of the receptor, respectively (Black et al., 1987), and had identical mobilities for wild-type and T⁻ myotubes. The other two bands, which were tentatively identified as the γ and δ subunits, both showed slightly increased mobilities in the T⁻ AChR relative to the wild-type AChR (Fig. 1), suggesting that the γ and δ subunits in T⁻ cells are different from those in wild-type cells. As two of the subunits have altered mobilities, a difference in covalent modification of the subunits, such as glycosylation, is more likely than a difference in peptide backbone.

To identify the AChR subunits unequivocally and to characterize further the difference between the two AChR preparations, AChRs from wild-type or T⁻ myotubes were partially purified by affinity chromatography on an α-BuTx-Sepharose column, subjected to SDS-PAGE, and analyzed by immunoblotting using antibodies specific for each of the four subunits of the muscle AChR (Gu and Hall, 1988*a*,*b*). Samples of the AChRs from wild-type and T⁻ myotubes were treated with glycosidases before immunoblotting to test for changes in N-linked glycosylation. Two glycosidases were used: endo H, which recognizes N-linked oligosaccharides with five or more mannose residues (high-mannose oligosaccharides) (Tarentino et al., 1978), and endo F, which cleaves all types of N-linked oligosaccharides, including both high-mannose and complex type oligosaccharides (Elder and Alexander, 1982).

The results of the immunoblotting experiment confirmed and extended those of the metabolic labeling experiment.

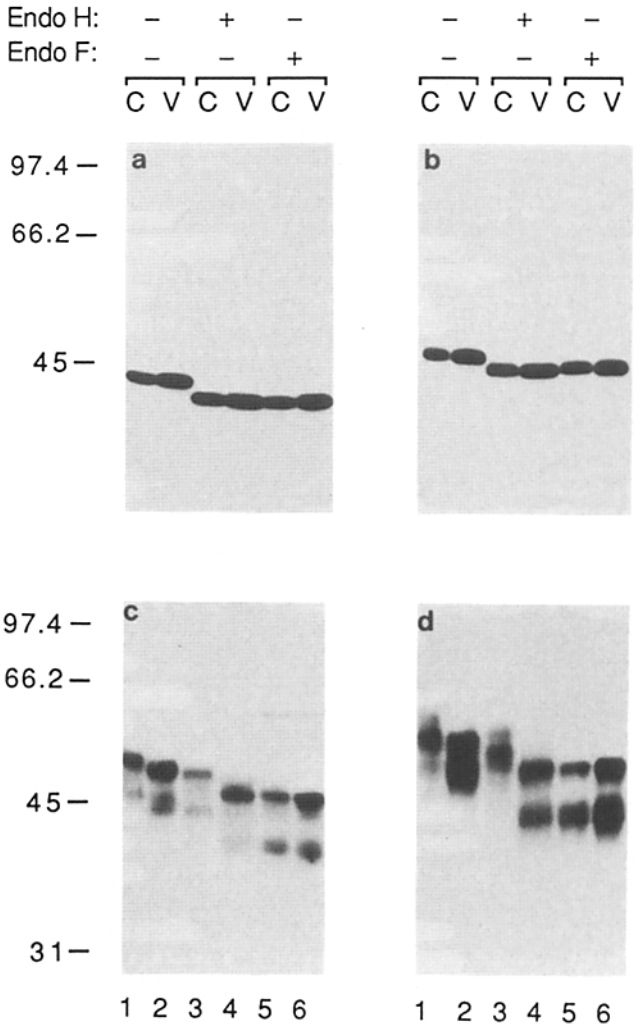

Figure 2. N-linked glycosylation of AChR in wild-type and T⁻ C2 myotubes. AChRs partially purified from wild-type control (lanes *C*, lanes *1*, *3*, and *5*) or T⁻ variant (lanes *V*, lanes *2*, *4*, and *6*) myotubes by affinity chromatography on α-BuTx-Sepharose column were either mock digested (lanes *1* and *2*), digested with endo H (lanes *3* and *4*), or endo F (lanes *5* and *6*) for 16 h at 37°C under conditions described in Materials and Methods. They were then subjected to reducing SDS-PAGE and immunoblotting analysis. Antibodies used as probes were (*a*) mAb 14-3-F-7 to detect α subunit, (*b*) mAb 124 to detect β subunit, (*c*) anti-γ 485 antibodies to detect γ subunit or (*d*) anti-δ 486 antibodies to detect δ subunit. The figure shows autoradiographs of the blots. Note that the size of γ and δ subunits are different in AChRs of wild-type and of T⁻ cells (*c* and *d*, compare lanes *1* and *2*) and that endo H digestion has different effects on their mobilities (*c* and *d*, compare lanes *3* and *4*). Numbers on the left correspond to molecular mass in kilodaltons.

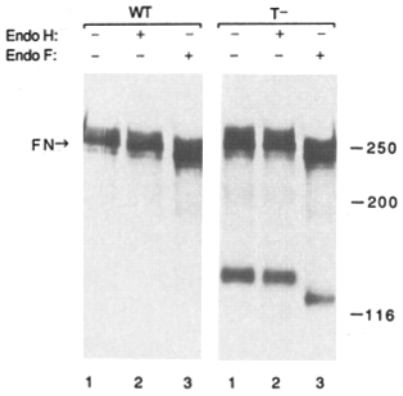

Figure 3. N-linked glycosylation of fibronectin in wild-type and T⁻ cells. Wild-type (*WT*) or T⁻ myotube cultures were incubated with [^{35}S]methionine and cysteine for 6 h. Fibronectin secreted into the medium by the cells was then isolated by affinity chromatography on gelatin-Sepharose column, mock digested (lanes *1*), digested with endo H (lanes *2*), or endo F (lanes *3*), and subjected to SDS-PAGE on a 5–10% gradient gel, followed by fluorography. The figure shows an autoradiograph that had been exposed for 12 h. Molecular mass standards on the right are laminin heavy chain (250 kD), laminin light chain (200 kD), and β-galactosidase (116 kD). *WT*, wild-type cells; *T⁻*, T⁻ variant cells; *FN*, fibronectin.

The mobilities of the single α band, identified with mAb 14-3-F7 (Dowding and Hall, 1987), were identical in wild-type and T⁻ AChR preparations (Fig. 2 *a*, lanes *1* and *2*). Treatment with endo H increased the mobilities of the α subunits equally in both preparations (Fig. 2 *a*, lanes *3* and *4*). Treatment with endo F also increased the mobilities of the α subunits, and to the same extent as did endo H (Fig. 2 *a*, lanes *5* and *6*). Thus, no difference was detected between wild-type and T⁻ α subunit either with or without endoglycosidase treatment. We conclude that the α subunit of the C2 AChR, like that of the rat muscle and *Torpedo* AChRs (Gu and Hall, 1988*b*; Nomoto et al. 1986), contains only high-mannose oligosaccharides and that the α subunits are indistinguishable in the AChRs of wild-type and T⁻ myotubes.

Similar results were obtained for the β subunit (Fig. 2 *b*). The β subunit, detected by mAb 124 (Gullick and Lindstrom, 1983), appears to contain only endo H-sensitive, high-mannose N-linked oligosaccharide residues, and no difference was found between wild-type and T⁻ AChR. In the case of the β subunit, the product of endo F digestion had a slightly higher apparent molecular weight than the product of endo H digestion (Fig. 2 *b*, compare lanes *3* and *4* with lanes *5* and *6*), as was seen with the β subunit of rat muscle AChR (Gu and Hall, 1988*b*) and with influenza virus HA protein (see Fig. 4). The reason for this is unknown.

Different results were obtained for the γ and δ subunits. First, two bands were observed for each subunit (Fig. 2, *c* and *d*). This pattern is similar to that seen on Western blots of the γ and δ subunits of rat muscle AChR under similar conditions, and is apparently due to proteolysis (Gu and Hall, 1988*b*). Second, as was found in the metabolic labeling experiment, the mobilities of both γ and δ subunits were increased in the T⁻ AChR compared to that of wild-type AChR (Fig. 2, *c* and *d*, lanes *1* and *2* in both). In each case, this difference was abolished by endo F treatment (Fig. 2, *c* and *d*, lanes *5* and *6* in both). Thus the difference in the electrophoretic mobilities of the γ and δ subunits in wild-type and T⁻ AChRs arises from a difference in N-linked glycosylation.

Further, a comparison of the products of endo H and endo F digestions shows that in the AChRs of wild-type cells, both γ and δ subunits have a mixture of endo H-sensitive (high-mannose) and endo H-resistant (complex) oligosaccharides. The γ and δ subunits from the T⁻ AChRs, in contrast, have only endo H-sensitive, high-mannose oligosaccharides. In the wild-type AChR, endo H treatment produced a small shift in the mobility of the γ subunit, and endo F treatment produced a larger shift (Fig. 2 *c*, lanes *1*, *3*, and *5*), whereas in the case of the T⁻ AChR, both endo H and endo F treatment produced the same shift in the γ subunit mobility (Fig. 2 *c*, lanes *2*, *4*, and *6*). Similar results were obtained for the δ subunit. Thus, the oligosaccharides in the γ and δ subunits in the T⁻ variant are defective in that they are not processed to complex, endo H-resistant forms as they are in wild-type cells.

Other Proteins in the T⁻ Variant Are Normally Glycosylated

The failure of T⁻ myotubes to process high-mannose oligosaccharides on the γ and δ chains of the AChR to complex forms could either be a general defect that affects all glycoproteins or one that is specific to the AChR. Two experimental approaches were taken to distinguish these possibilities. First, we compared the pattern of sensitivity of wild-type and T⁻ variant cells to various lectins, a technique commonly used to detect defects in glycosylation of surface proteins in other types of variant cell lines (Stanley, 1984; Kingsley et al., 1986). No significant differences were seen in the pattern of susceptibility of the two cell types to wheat germ agglutinin, ricin, and concanavalin A (data not shown), suggesting that T⁻ cells do not have a general defect in glycosylation of surface proteins.

Next we examined the N-linked glycosylation of two specific proteins, fibronectin, an endogenous extracellular matrix protein that is secreted into the medium of myocytes, and influenza virus HA protein, a foreign membrane protein that is expressed on the surface of cells infected with influenza virus. Fibronectin was isolated from the media of wild-type or T⁻ myotube cultures that had been labeled with [^{35}S]methionine and cysteine for 6 h. In preparations from both wild-type and T⁻ cultures, endo H produced little or no shift in the electrophoretic mobility of fibronectin (Fig. 3, lanes *2*), while endo F treatment produced a distinct shift (Fig. 3, lanes *3*). No difference was observed under any condition in the mobilities of fibronectin in wild-type and T⁻ cells.

The fibronectin preparation obtained from T⁻ cultures also contained a protein of 135,000 M_r that was not detectable in wild-type cultures (Fig. 3). Although we do not know the identity of this protein, its behavior after glycosidase treatment clearly indicated that it also contained endo H-resistant oligosaccharides.

Similar results were also obtained with influenza HA protein expressed in wild type and T⁻ cultures after infection with influenza virus. In both cases, HA proteins remained endo H-sensitive shortly after synthesis (Fig. 4 *A*), but be-

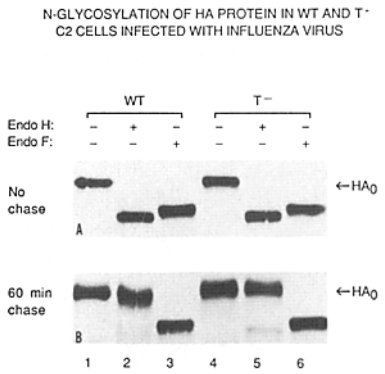

Figure 4. N-linked glycosylation of hemagglutinin in wild-type and T⁻ cells. Wild-type (lanes *1–3*) or T⁻ (lanes *4–6*) myotube cultures were infected with influenza virus and pulse labeled with [^{35}S]methionine/cysteine for 10 min as described in Materials and Methods. Cell extracts were then prepared either immediately after the pulse (*A*) or after a 60-min chase in the presence of excess unlabeled amino acids (*B*) and immunoprecipitated with an antiserum raised against the virus. Immunoprecipitates were either mock-digested (lanes *1* and *4*), digested with endo H (lanes *2* and *5*), or endo F (lanes *3* and *6*), analyzed on 9% SDS polyacrylamide gels under reducing conditions, and fluorographed. Control experiments showed that all the HA protein was intracellular at the end of the pulse and that ~50% reached the cell surface during the 60-min chase in both cell types.

came endo H–resistant after a 60-min chase (Fig. 4 *B*). These results, taken together with those on fibronectin, indicate that T⁻ cells are capable of producing complex N-linked sugars, and that the alteration in oligosaccharides on the γ and δ chains of the AChR is not a general defect.

Surface AChRs in T⁻ Cells Contain Normal N-linked Sugars

Are the oligosaccharides on the surface AChRs of T⁻ cells sensitive to endo H? Surface AChRs were isolated from wild-type and T⁻ myotubes by labeling intact myotubes with α-BuTx, followed by extraction and immunoprecipitation with anti–α-BuTx antibodies. Immunoblots of the AChRs were then made using anti–γ 485 antibodies (Gu and Hall, 1988*a*,*b*) as the probe. The results (Fig. 5) show that the γ subunit of surface AChRs derived from T⁻ myotubes, like those from wild-type myotubes, contain a mixture of endo H–sensitive and endo H–resistant forms of N-linked sugars. Thus the surface AChRs in T⁻ cells have normal N-linked sugars.

Immunofluorescent Localization of Internal AChR

The localization of the internal AChR in wild-type and T⁻ myotubes was investigated by immunofluorescence. Myotube cultures were incubated with unlabeled α-BuTx to block surface AChRs, then fixed, permeabilized, and stained with rho-α-BuTx as described in Materials and Methods. In parallel experiments cultures of myoblasts and myotubes were stained with Golgi or ER markers. A monoclonal antibody against BiP (Bole et al., 1986) was used for the ER, and both wheat germ agglutinin and a Golgi-specific monoclonal antibody (Allan and Kreis, 1986) for the Golgi apparatus. Similar results were obtained with both Golgi markers.

In both myoblasts and myotubes stained with anti–BiP (Fig. 6, *A* and *B*), the ER forms an extensive network throughout the cell. Details of the network can be observed toward the edge of the myoblasts, where the cell is thinner. The pattern of anti–BiP staining is similar to that observed with lipid dye markers (Terasaki et al., 1984), but differs somewhat from that observed with antibodies to membrane fractions derived from the rough ER (Louvard et al., 1982). The most conspicuous difference is the bright perinuclear staining seen in the latter case. The difference in staining could occur because BiP is a luminal protein, rather than a membrane protein.

As observed by others, the Golgi pattern was strikingly different in myoblasts and myotubes (Tassin et al., 1985) (Fig. 6, *C* and *D*). In myoblasts the Golgi apparatus was close to the nucleus and highly polar. A pattern of coarse dots was observed instead of the finely granular texture seen for the ER. In myotubes, evenly distributed but discontinuous Golgi staining was seen around each nucleus, and isolated dots of staining that seemed to link the Golgi apparatus from neighboring nuclei were distributed throughout the cytoplasm.

In wild-type myotubes the pattern of internal AChR staining showed characteristics of both ER and Golgi (Fig. 7). The most prominent feature was the continuous perinuclear staining, which along with the diffuse staining throughout the cytoplasm, resembled that seen with ER markers. Coarsely dotted staining, like that of the Golgi apparatus was also seen, however. In double-label experiments, such dots were seen to be stained not only by rho-α-BuTx, but also by the anti-Golgi antibody (Fig. 8).

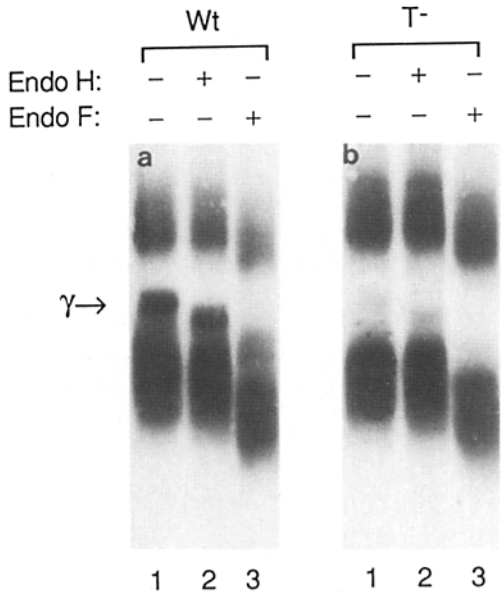

Figure 5. N-linked glycosylation of the γ subunit of surface AChR in wild-type and T⁻ cells. Wild-type (*Wt*) or T⁻ myotube cultures were incubated with α-BuTx to label the surface receptor. The cells were then lysed and surface AChR isolated by immunoprecipitation with anti–α-BuTx antibodies as described in Materials and Methods. Immunoprecipitates were either mock-digested (lanes *1*), digested with endo H (lanes *2*), or endo F (lanes *3*), and separated on SDS gel and processed for Western blot analysis, using anti–γ 485 antibodies as the probe. The figure shows an autoradiogram of the blot. Arrow indicates the position of the γ subunit on the gel.

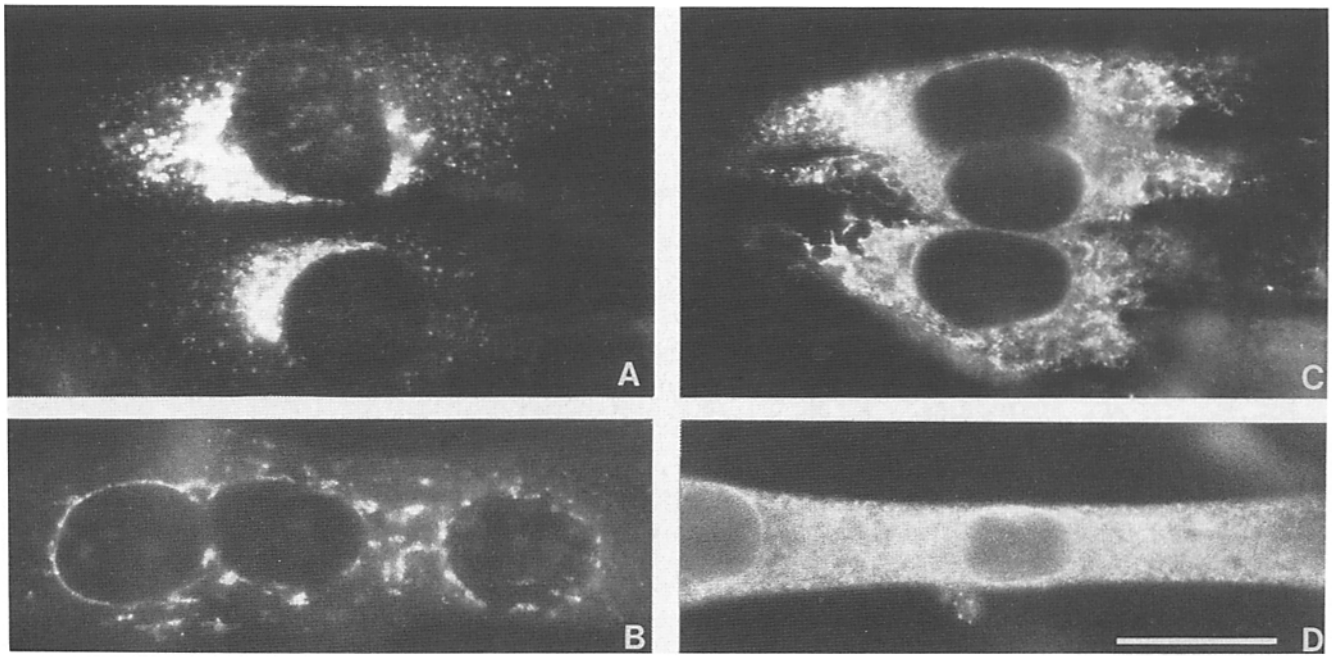

Figure 6. Immunofluorescent staining of C2 cells with Golgi and ER markers. Wild-type C2 myoblasts (*A* and *C*) or myotubes (*B* and *D*) grown on glass coverslips were fixed and permeabilized as described in Materials and Methods. They were then stained with an anti-Golgi (*A* and *B*) or with an anti-BiP (*C* and *D*) monoclonal antibody, followed by fluorescein- (FITC) conjugated anti-mouse antibodies. A similar staining pattern was obtained when T^- cells were used. Bar, 20 μm.

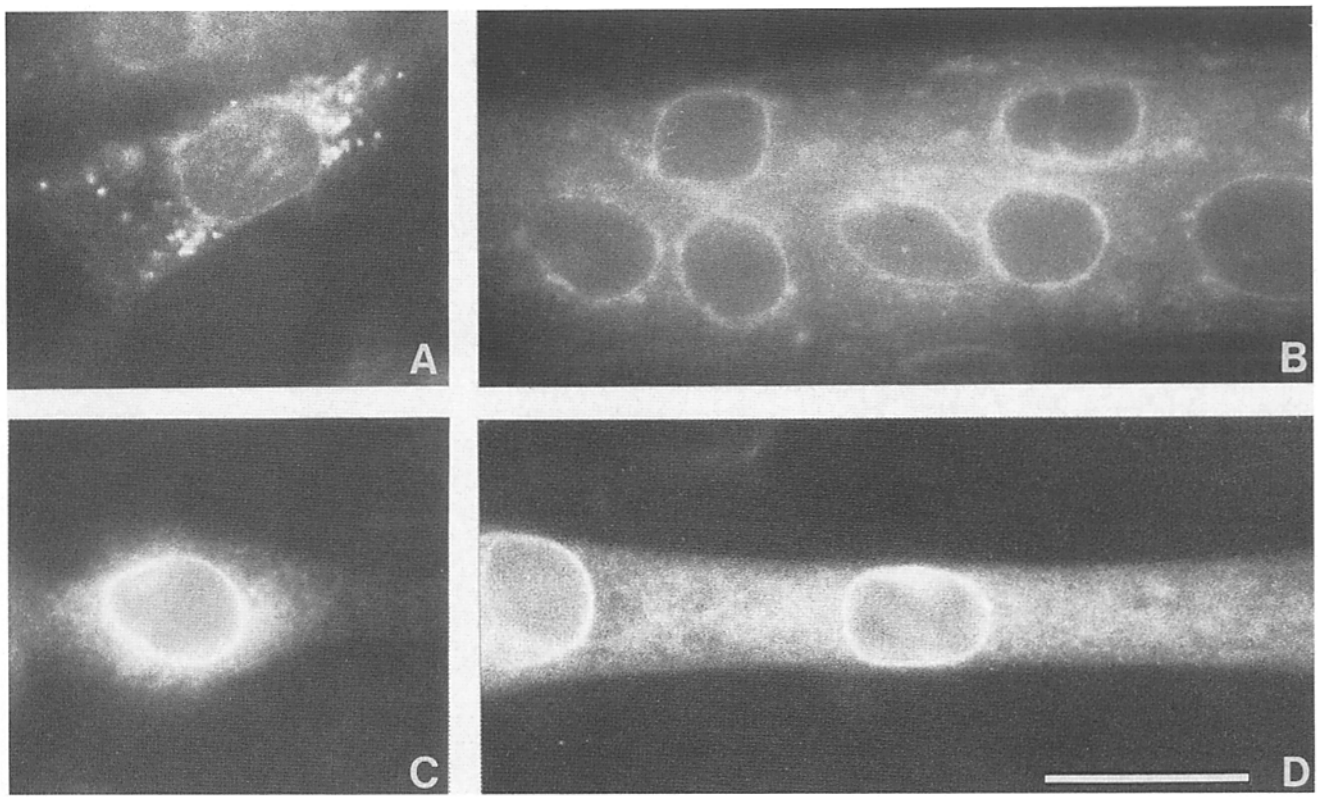

Figure 7. Immunocytochemical localization of intracellular AChRs in wild-type and T^- cells. Wild-type (*A* and *B*) and T^- (*C* and *D*) C2 cells were grown on glass coverslips and switched to fusion medium. After 3 d in fusion medium, cell surface AChRs were blocked with unlabeled α-BuTx. The cells were then fixed, permeabilized, and stained with rho-α-BuTx as described in Materials and Methods. Although C2 myoblasts in growth medium do not show any staining after this procedure (not shown), C2 cultures in fusion medium include mononucleated cells that do express AChR. The figure shows mononucleated cells (*A* and *C*) and multinucleated myotubes (*B* and *D*). Bar, 20 μm.

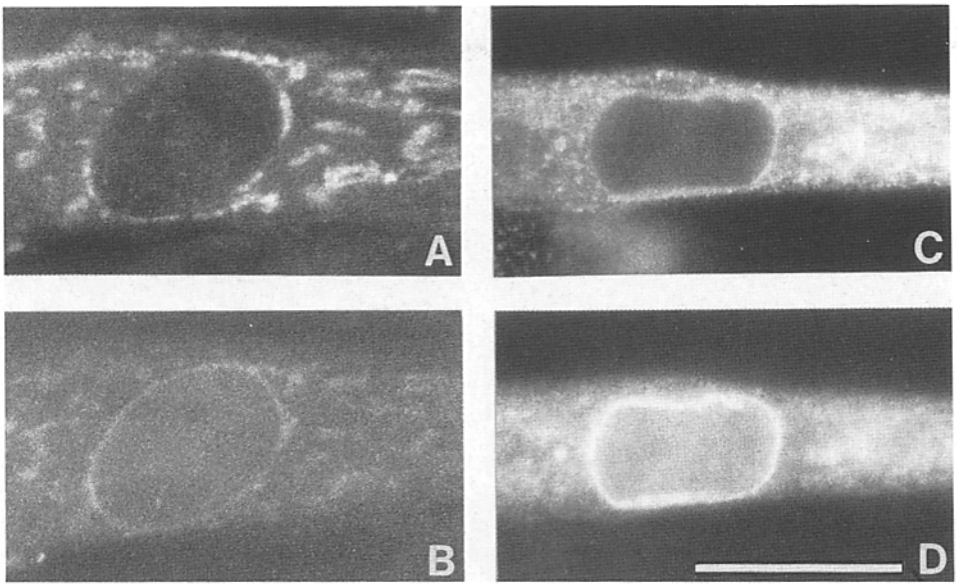

Figure 8. Simultaneous localization of AChR and Golgi apparatus or ER in C2 myotubes. Wild-type (*A* and *B*) or T⁻ (*C* and *D*) cells kept for 3 d in fusion medium were stained with rho-α-BuTx (*B* and *D*) as described in the legend to Fig. 7 and, simultaneously, with an anti-Golgi (*A*) or anti-BiP (*C*) monoclonal antibody, followed by fluorescein-labeled second antibodies. Bar, 20 μm.

In T⁻ myotubes, the pattern of staining more closely resembled that of the ER. A bright, continuous perinuclear staining was seen, along with a diffuse, finely textured staining in the cytoplasm. No evidence was found for the coarse, dot-like pattern of the Golgi staining (Fig. 7).

The distinction in rho-α-BuTx staining pattern in wild-type and T⁻ cells was most clearly demonstrated in myoblasts. Although C2 myoblasts make little, if any, AChR when cultured under growth conditions, occasional mononucleated cells in fusion medium showed intense intracellular staining with rho-α-BuTx. In wild-type cultures, this staining was near the nucleus and was distinctly polar, resembling that seen with Golgi markers; in T⁻ cultures, in contrast, the staining was evenly distributed throughout the cytoplasm (Fig. 7).

Localization of the Internal AChR by Subcellular Fractionation

As an additional method of localizing the internal pool of AChR in wild-type and T⁻ cells, we used a sucrose gradient centrifugation procedure recently developed by Smith et al. (1987) that separates membranes derived from the Golgi apparatus and those from the ER. When applied to C2 myotubes cultures, these methods gave good separation between the Golgi marker enzyme, mannosidase II (Novikoff et al., 1983), and a marker for the endoplasmic reticulum, glucose-6-phosphatase (Farquhar et al., 1974) (Fig. 6, *B* and *D*); another Golgi marker, UDP-galactose/*N*-acetylglucosamine galactosyltransferase, which is located in the *trans*-Golgi (Roth and Berger, 1982; Slot and Geuze, 1983), sedimented to a density that was slightly higher than that of mannosidase II, in agreement with the results of Smith et al. (1987). Although the gradients showed little or no contamination of Golgi fractions by the ER marker enzymes, there was a small peak of activity of both Golgi markers in the fractions containing ER markers. Surface membrane, identified by bound ^{125}I-α-BuTx, was also found at approximately the same position in the gradient as glucose-6-phosphatase, the ER marker (data not shown). Thus the fractions containing ER are heterogeneous.

To examine the internal AChR, myotube cultures were incubated with unlabeled α-BuTx to block surface receptors, then homogenized, and subjected to sucrose gradient centrifugation. Gradient fractions were analyzed for AChR by ^{125}I-α-BuTx binding, and for ER and Golgi markers as described in Materials and Methods. In homogenates from wild-type myotube cultures, the major peak of AChR was found in a position coincident with the ER marker, glucose-6-phosphatase (Fig. 9, *a* and *b*). A smaller peak was also detected in the fractions containing the Golgi markers. Analysis of T⁻ homogenates, however, showed that virtually all of the AChR was in the region of the gradient containing ER membranes (Fig. 9, *c* and *d*). Little, if any, AChR was detected in the Golgi region. These experiments demonstrate clearly that the internal AChR in T⁻ myotubes is not accumulated in the Golgi apparatus, and suggest that it may be retained in the ER.

Discussion

We have compared the properties and intracellular location of AChRs in wild-type C2 muscle cells and in T⁻, a C2 variant that accumulates AChR in an intracellular pool. The principal conclusion of our experiments is that in wild-type cells AChR is present both in the Golgi apparatus and in the ER; T⁻ cells, in contrast, have reduced levels of AChR in the Golgi apparatus, and accumulate it in the ER. As shown elsewhere, inefficient transport from the ER to the Golgi apparatus is not a general defect in T⁻ cells, but appears to be specific for the AChR. Thus T⁻ myotubes have on their surface normal amounts of both the insulin receptor and the transferrin receptor and, after infection with influenza virus show normal transport of hemagglutinin protein to the surface (Gu et al., 1989; see also Fig. 4 legend).

In normal myotubes ~30% of the total AChR is intracellular, most of which serves as precursor for receptor on the surface (Devreotes et al., 1977; Gu et al., 1989). Fambrough and Devreotes (1978) localized intracellular AChR to the Golgi, using ultrastructural methods. Recent pulse-labeling

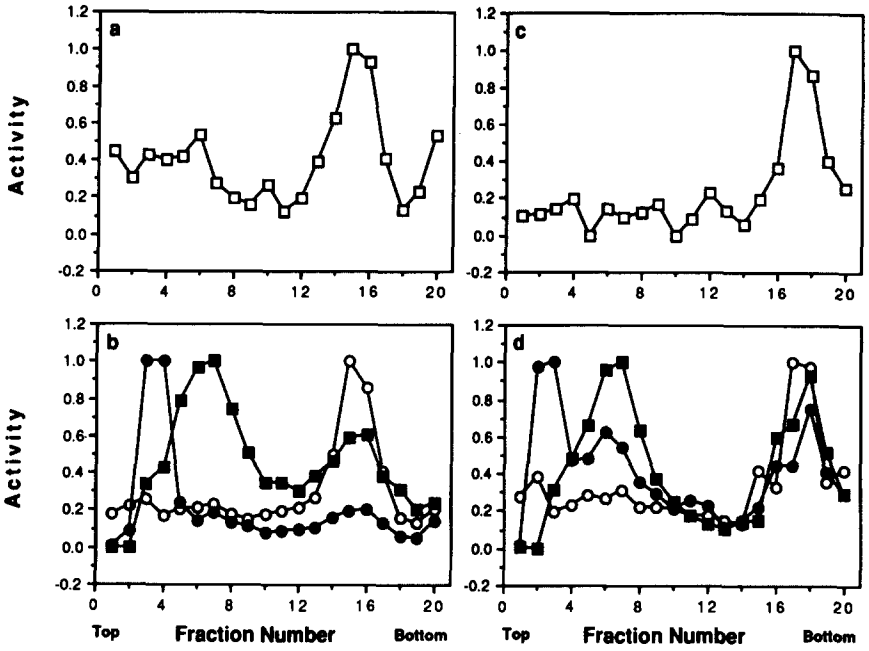

Figure 9. Subcellular localization of AChR in wild-type and T⁻ myotubes. Myotube cultures of wild-type (*A* and *B*) or T⁻ (*C* and *D*) cells were incubated with excess unlabeled α-BuTx to block surface AChR. The cells were then homogenized and centrifuged on 20–69% sucrose gradients as described in Materials and Methods. 20 200-μl fractions were collected from each gradient and assayed for toxin-binding activity and for markers of the endoplasmic reticulum and the Golgi apparatus. The data were normalized to the highest activity for each marker. *a* and *c*, toxin-binding activity (□); *b* and *d*, glucose-6-phosphate activity (○), mannosidase II activity (●) and UDP-galactose/*N*-acetylglucosamine galactosyltransferase activity (■).

experiments, followed by subcellular fractionation, have shown that assembled AChR is also present in the ER (Smith et al., 1987). Our results on wild-type C2 cells confirm both of these findings. Subcellular fractionation shows that most of the intracellular AChR is in fractions that contain membranes from the ER, but that a small peak of AChR is also in fractions containing Golgi membranes (Fig. 9). Immunocytochemical experiments also show distinct staining for the AChR in the Golgi apparatus, as well as a more diffuse staining that is characteristic of the ER. Although more prominent in the Golgi than in the ER when viewed by immunocytochemistry, most of the intracellular AChR in C2 myotubes is probably in the ER. The subcellular fractionation results are consistent with this distribution, as is the finding that most of the internal AChR in wild-type cells contains only endo H-sensitive sugars (Gu, Y. and Z. W. Hall, unpublished observations).

In contrast to wild-type cells, most of the AChR in T⁻ myotubes is intracellular and does not serve as precursor for surface receptor (Gu et al., 1989). Virtually all of the internal AChR appears to be in the ER. No AChR was observed in the Golgi fractions after subcellular fractionation (Fig. 9); the more sensitive immunocytochemical experiments also failed to detect AChR in the Golgi apparatus. In neither myoblasts nor myotubes was there any sign of the coarsely dotted staining pattern that is characteristic of the Golgi apparatus.

Analysis of N-linked glycosylation of the AChR subunits in the T⁻ variant further indicated that they were accumulated in a pre-Golgi compartment. Both γ and δ subunits of the internal AChR in the T⁻ variant lacked endo H-resistant oligosaccharides. Because the processing of N-linked sugars from high-mannose, endo H-sensitive forms to complex, endo H-resistant forms occurs in the Golgi apparatus (Kornfeld and Kornfeld, 1985), AChR that is retained in the ER would necessarily lack endo H-resistant oligosaccharides. Accumulation of fully assembled AChR in the ER of T⁻ myotubes is consistent with the earlier finding by Smith et al. (1987) that the AChR is assembled there.

Our results are most consistent with AChR localization in the ER of T⁻ myotubes, although the possibility that the receptor accumulates in a closely related compartment cannot be ruled out. The pattern of AChR staining in T⁻ cells strongly resembles the pattern seen with anti-BiP, but is not identical with it. First, the AChR staining in T⁻ cells was often more finely textured than anti-BiP staining. Second, the most prominent feature of the AChR staining, the intense perinuclear ring, was only occasionally seen with anti-BiP. Perinuclear ER is observed with the electron microscope, however, and other ER antibodies show strong perinuclear staining (Louvard et al., 1982). Thus, an alternative explanation for the discrepancy is that the pattern seen with anti-BiP, which is a luminal protein, may not reflect the complete distribution of ER membranes.

What is the primary defect in T⁻ cells? Although a number of genetic variants of mammalian cell lines that lack glycosylation processing enzymes have been described (Stanley, 1984; Kingsley et al., 1986), the T⁻ variant does not appear to be one of these. First, our experiments show no evidence for a general defect in glycosylation. Lectin sensitivity, and the processing of fibronectin and influenza virus HA protein appear to be unchanged in T⁻ cells. Second, the T⁻ phenotype cannot be reproduced in wild-type cells by inhibitors of enzymes in the glycosylation pathway (Gu, Y. and Z. W. Hall, unpublished results).

A transport-defective mutant of CHO cells, gro29, has also been described by Tufaro et al. (1987), but the properties of this variant are different from those of the T⁻ variant of C2 cells. In gro29 cells, both oligosaccharide processing and export of viral protein is defective and protein accumulates in or near the Golgi apparatus. In T⁻ cells, in contrast, viral protein is processed and transported to the surface with normal kinetics.

The defect in T⁻ cells is not a general one, and may be specific to the AChR. The latter possibility raises the question of whether the defect is in the AChR itself or is in another protein. This question is unresolved by our experiments. A previous finding that the α subunit is less rapidly degraded in T⁻ than in wild-type cells (Gu, Y., manuscript submitted for publication) could be explained by a change in primary sequence of the α subunit, but could also have other explanations.

One of the most puzzling aspects of our experiments is that a minor proportion of the AChRs in T⁻ cells is normally glycosylated (Fig. 5) and is transported to the surface with normal kinetics (Gu et al., 1989). One possible explanation is that genes coding for the α subunit on both chromosomes are expressed. It is possible that two copies of the α subunit are produced, one of which is mutated. If AChRs were assembled randomly, then only a minority of AChRs would have two correct α subunits. Another possibility is that one of the subunits undergoes a conformational change before assembly such that incorrect folding, which is normally a rare event, becomes more frequent after a mutational alteration. The incorrectly folded subunit would then be assembled to form a defective AChR. We have shown that internal AChRs bind α-BuTx and have a normal sedimentation coefficient (Gu et al., 1989), but their properties are otherwise unexamined.

Such suggestions imply that transport mechanisms can distinguish between authentic and defective AChRs. Numerous examples are available of defective or unassembled proteins produced by mutation or by recombinant techniques that do not exit the ER (e.g., Leitersdorf et al., 1988; Doms et al., 1988; Gething et al., 1986; Doyle et al., 1985, 1986). In some cases these have been shown to be complexed to a 78-kD protein, BiP, found in the ER lumen (Haas and Wabl, 1983; Bole et al., 1986; Munro and Pelham, 1986; Gething et al., 1986). In preliminary experiments, we found no evidence for AChR-BiP complexes in T⁻ cells. BiP is not precipitated by antibodies to the AChR, nor do anti-BiP antibodies precipitate the α subunit of the AChR (Gu, Y. and Z. W. Hall, unpublished data). The retention of the AChR in the ER is thus apparently not due to its association with BiP.

A final possibility arises from the observation that myotubes normally have a stable internal pool of AChRs that does not serve as precursor for surface receptor (Devreotes et al., 1977; Gu et al., 1989). The function and location of this pool is unknown, but it could reflect the ability of the cell to regulate the proportion of AChR that is on its surface. If so, regulation in the T⁻ variant might be altered so that receptor is preferentially retained. An intriguing feature of such a scheme would be the possibility of regulating transport from the ER to the Golgi apparatus to achieve different densities of surface AChR.

We thank Drs. J. Lindstrom, S. Froehner, V. Allan, and D. Bole for generous gifts of antibody; and Dr. J. White for providing us with influenza virus and antibody. We are also grateful to Dr. R. Kelly for many helpful discussions and to members of the laboratory for their useful comments on the manuscript.

This work was supported by grants from the National Institutes of Health and the Muscular Dystrophy Association.

Received for publication 22 February 1989 and in revised form 7 April 1989.

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